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Blancafort, Pilar

INTRODUCTION

It is perhaps surprising that most of the cells within the bulk of a breast tumor are differentiated cells, which indeed do not have tumorigenic potential at all. The Tumor Initiating Cells (TIC) comprise a small population that can generate tumors with very small amounts (for example, as little as 100 cells) once injected in immunodeficient mice (1-4). These cells, named Cancer Stem Cells (CSCs), are able to sustain self-renewal ability *in vitro* and have tumorigentic capabilities. CSCs could be visualized as "corrupted" versions of normal stem cells. Because of their ability to self-renew during a lifetime of an individual, these cells could be primary targets of transformation, by acquisition of genetic defects (for example, mutations in tumor suppressor genes and oncogenes) but also by acquiring transcriptional and epigenetic aberrations (3-4). Like their normal stem cell counterparts (5), CSCs are believed to naturally overexpress proteins in the surface that extrude DNA-damaging agents (like the ones used in chemo-therapy for breast cancer) possibly as natural mechanism of stem cells to protect the integrity of their long-life genomes (6-7). Because of their ability to initiate a tumor, their potential to migrate, disseminate and differentiate, and their intrinsic resistance to chemotherapeutic agents, CSCs are primordial targets in breast cancer therapeutics. Recently, the triple negative breast cancer subgroup or basal-like breast cancer, associated with the poorest prognosis in breast cancer patients, has been found enriched in CSCs (8,9) *Thus, we are in a critical need for the development of novel technologies to detect and specifically target CSCs, in order to suppress the intrinsic growth capabilities of the tumor*.

Our objective is to develop novel technologies to target genes differentially expressed in CSCs, which play a role in maintaining self-renewal and tumorigenicity. We have recently found that the primordial embryonic transcription factor genes, such as SOX2, responsible for self-renewal of embryonic stem cells (10), are up-regulated in basal-like breast cancer patients, and that their overexpression is associated with poor prognosis (11). The structure of the chromatin in these self-renewal gene promoters is a major determinant associated with transcriptional dysregulation and oncogenesis. Chromatin structure and function is controlled in large part by the post-translational modification of histones and the incorporation of specialized histone variants into nucleosomes (12). Strikingly, histone proteins are highly modified by an array of diverse post-translational modifications, including acetylation and methylation (12). The large number of modifications and the ability of individual histones to be multiply modified has given rise to the idea that a "histone code" may exist that acts in a combinatorial manner to elicit distinct biological effects (13-14). This code is thought to function through the physical alteration of chromatin structure and/or through the recruitment of effector proteins to the sites of histone modification (12). Several histone modifications have been linked to oncogenesis and cancer stem cell biology (15-18). Histone H3 lysine 9 (H3K9) methylation, for example, is critical for gene repression and heterochromatin formation through the recruitment of heterochromatin protein 1, which binds the methyllysine residue (19-21). In contrast, the methylation of H3 lysine 4 (H3K4) is linked to transcriptional activation via the recruitment of TFIID and several chromatin-remodeling and modifying enzymes (22-24). Histone methylation was once considered to be irreversible, however, recent identification of lysine-specific histone demethylases (KDMs) has revealed histone methylation to be a dynamically regulated process (25-26). KDMs contain a JmjC domain, a signature motif conserved from yeast to humans (27). To date, there have been a number of H3K4 demethylases identified, including PLU-1 (JARID1B) (25-26). H3K4 demethylation can help maintaining a repressed chromatin state (28). The diversity of chromatin "editing" enzymes underscores the importance of

the epigenetic landscape in controlling gene expression. Further, our ability to stably alter gene expression states via epigenetic reprograming is likely to have far reaching implications for controling human deseases including breast cancer (15, 29). This Idea Award proposal aims to specifcally test the utility of targeting the enzymes that methylate or demethylate these lysine residues in order to shut down the expression of genes that promote breast cancer stem cell renewal. We propose the construction of novel factors named Designed Epigenetic Remodeling Factors (DERFs). DERFs will be targeted to specific self-renewal promoters using engineered arrays of six-Zinc Finger (ZF) domains, which target 18-bp sites and potentially have unique specificity in the human genome. The result of this work should lead to the generation of novel chromatin remodeling factors targeting CSC self-renewal and tumorigenicity.

BODY

YEAR 2:

Obtained Results and Discussion (Blancafort Lab)

We used a second breast cancer cell line (MCF7) to confirm silencing activity of the two most powerful ATFs binding in close proximity to the transcriptional start site (ZF-552SKD and ZF-598SKD), as shown in Fig. 1A. MCF7 cells were stably transduced with the 552SKD or 598SKD construct and the ATF's expression was controlled by doxycycline (Dox). By quatitative real-time PCR and western blot analysis, we demonstrated the potential of our ATFs ZF-552SKD and ZF-598SKD to down regulate SOX2 upon Dox induction (+Dox) by mRNA and protein levels (Figure 1A and B).

All our ATF constructs are designed with a C-terminal HA-tag to monitor ATF expression upon Dox induction. In Fig 1C immunoflourescence shows the expression of the HA-tag after ATF induction using Dox (+Dox) but not before (-Dox). Next, we

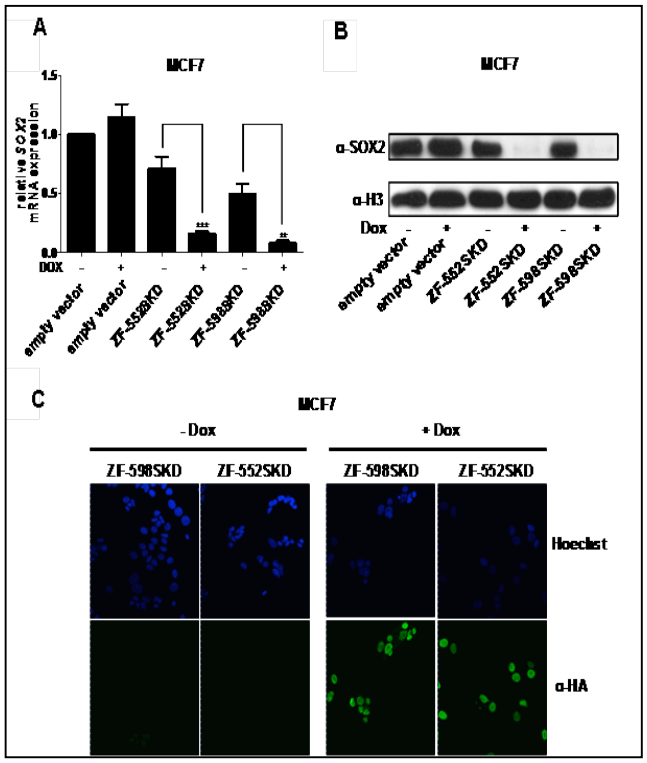

Fig. 1. **(A)** Quantification of *SOX2* mRNA by qRT-PCR in MCF7 breast cancer cells. MCF7 cells were stable transfected with empty vector control, ZF-552SKD and ZF-598SKD. The ATF expression was induced by Doxycyclin and is indicated in the x-axis (+/- Dox). Error bars show S.D and statistical significance was analysed using student t-test (*** p<0.001, ** p<0.01). **(B)** Quantification of SOX2 protein by Western blot in MCF7 cells. Histone H3 is used as a loading control. Samples are the same as in (A). **(C)** Immunofluorescence analysis of transduced MCF7 (ZF-552SKD and ZF-598SKD). Detection of ZF-552SKD and ZF-598SKD (green, α HA-tag) and nuclear staining was performed using Hoechst (blue). The left panel shows non-induced MCF7 cells (no Dox) and the right panel shows induced MCF7 cells (Dox). Images are taken at 40x.

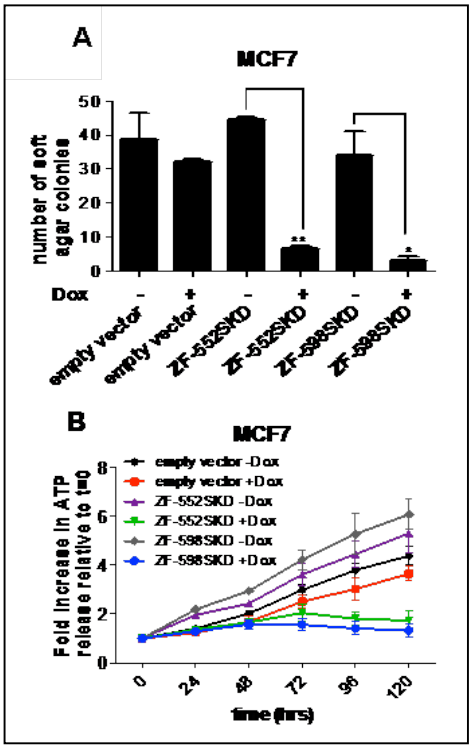

Fig. 2. Upper panel: Quantification of colony numbers in soft agar. Shown are results from MCF7 cells transduced with empty vector, ZF-552SKD and ZF-598SKD uninduced (no Dox) and induced (Dox) cells. Error bars represent S.D. Statistical significance was analysed by t-test (** p<0.01, * p<0.05). Lower panel: Proliferation analysis of MCF7 cell. Empty vector, ZF-552SKD and ZF-598SKD transduced MCF7 cells were induced with Doxycyclin every 48hrs. Uninduced cells were used as controls. The y-axis indicates fold increase in ATP release relative to time point 0 measured by luminescence. Statistical significance was analysed using 2-way ANOVA with p-value < 0.001 at the last time point

analyzed whether the MCF7 cell lines stably expressing the ZF-552SKD and ZF-598SKD were able to decrease cell proliferation and anchorage independent growth. Both constructs demonstrated a strong inhibition of anchorage independent growth and cell proliferation after Dox-induction (+Dox) as compared with no Dox control (-Dox) (Fig 2). Binding of the two ATFs to their specific target site within the SOX2 promoter was confirmed by chromatin immunoprecipitation (ChIP) using an anti HA-tag antibody in Dox-induced (+Dox) and un-induced (-Dox) MCF7 cells stably transduced with ZF-552SKD or ZF-598SKD (Fig. 3).

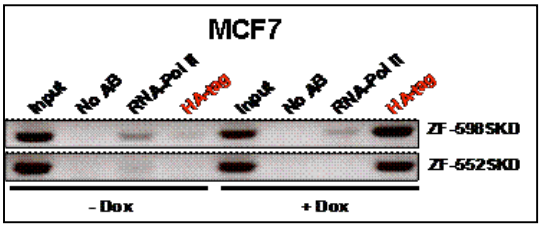

Fig. 3. Chromatin immunoprecipitation analysis. MCF7 cells stable transduced with ZF-552SKD and ZF-598SKD were subjected to ChIP analysis before and after induction of ZF expression by Sox (+/-Dox)

After these cell culture analysis of our ZF-constructs, we used the MCF7 cell line stably transduced with the ZF-598SKD construct in a xenograft mouse model of breast cancer to investigate whether our ATF inhibits tumor cell growth *in vivo*. ATF expression was induced 21 days after injection at a tumor size of approximately 0.5cm (Fig.4A). Tumor growth in vivo was reduced when ATF expression was induced by administration of Dox (ZF-598SKD +Dox) as compared with empty vector +Dox and un-induced ATF (ZF-598SKD –Dox) respectively (Fig. 4B and C).

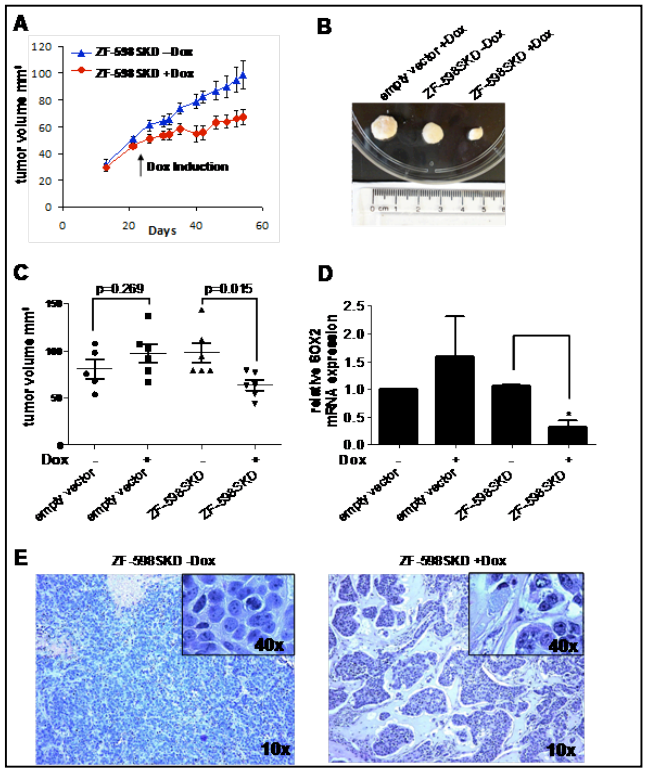

Fig. 4. A SOX2-specific ATF inhibits the growth of pre-existing s.c xenografts of MCF7 cells. (A) Time course plot of tumor volume monitored by caliper measurements. Animals (N=6) were either maintained in a Dox-free diet (-Dox) or induced with Dox diet (arrow) at day 21 post-injection. (B) Picture of representative tumors collected at day 28 post-induction from induced empty vector, un-induced ZF-598SKD, and induced ZF-598SKD animals. (C) Tumor volume measurements at day 21 post-induction from empty vector and ZF-598SKD groups (N=6 animals per group). Differences between groups were assessed by a Wilconxon rank sum test. (D) Quantification of SOX2 mRNA expression by qRT-PCR in tumor samples from a representative tumor xenograft. Bar graphs represent the mean and SD of three tumor samples. Differences in gene expression were calculated with a Student's t-test, *P=0.01 (E) Hematoxylin-Eosin staining of representative ZF-598SKD –Dox and +Dox tumor sections. Un-induced (–Dox) animals revealed highly compact tumors. Induced (+Dox) ZF-598SKD sections comprised discrete islands of tumor cells, separated by intervening stroma. Pictures were taken at 10x and a detail of a 40x magnification is shown.

At day 49 post-injection animals were sacrificed and SOX2 expression was analyzed in the extracted tumors. In tumors where ATF expression was induced with Dox a decrease in SOX2 mRNA expression was detected. Furthermore, immunohistochemistry analysis of untreated tumors (-Dox) and treated

tumors (+Dox) revealed a higher density of closely packed tumor cells in the tissue of untreated tumors (-Dox) as compared with Dox-treated (+Dox) tissue.

Next, we generated ***Designed Epigenetic Remodeling Factors (DERFs)***. We fused the catalytic active domain of the DNA methyltransferase 3a (DNMT3a) and the catalytic mutant DNMT3aE74A to the ZF-552 and the ZF-598 to obtain the fusion proteins ZF-552DNMT3a, ZF-598DNMT3a, ZF-552DNMT3aE74A and ZF-598DNMT3aE74A. These constructs were used for the stable transduction of MCF7 cells and expression of the ZF-constructs was properly induced by Dox treatment. Down-regulation of SOX2 expression was confirmed by RT-PCR and western blot analysis (Fig.5A and B). In RT-PCR, we compared the down-regulation effect of the ZF-552DNMT3a construct with the same DNA-binding domain fused to the SKD (ZF-552SKD). Figure 5A shows that the ZF-552DNMT3a construct significantly down-regulates SOX2, but to a lesser extent than the SKD construct. However, our proliferation analysis revealed that upon Dox-removal after 48hrs, the ZF-552SKD and ZF-598SKD constructs resumed their proliferation after 144 hrs, while the DNMT3a constructs show long-lasting proliferation inhibition even after 8 days (Fig. 5C).

A possible explanation to this effect could be direct DNA-methylation introduced by our DNMT3a constructs locks SOX2 expression and therefore represses SOX2 expression more permanent than the SKD constructs, resulting in a stable decrease of cell proliferation. To proof whether the ZF-552DNMT3a and ZF-598DNMT3a repressed SOX2 expression by means of DNA-methylation, we performed DNA methylation studies by sodium bisulfate conversion of the genomic DNA, followed by MASSArray spectroscopy. The methylation level of individual CpG islands was examined in our stably transduced MCF7 cell lines after Dox-treatment in two regions of the SOX2 promoter and compared with un-transduced MCF7 cells (Fig 6). The expression of both DERFs (ZF-552DNMT3a and the ZF-598DNMT3a) in MCF7 cells resulted in a higher density of DNA-methylation (up to 100%) in both regions analyzed, as compared with their catalytic death mutants (ZF-

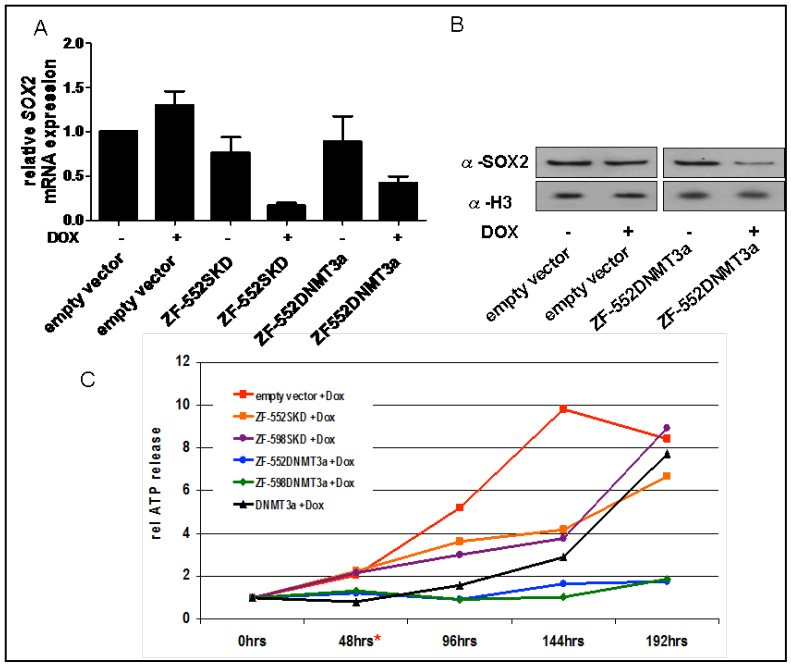

Fig. 5. **(A)** Quantification of *SOX2* mRNA by qRT-PCR in MCF7 breast cancer cells. MCF7 cells were stable transfected with empty vector control, ZF-552SKD and ZF-552DNMT3a. The ATF expression was induced by Doxycyclin and is indicated in the x-axis (+/- Dox). Error bars show S.D **(B)** Quantification of SOX2 protein by Western blot in MCF7 cells. Histone H3 is used as a loading control. Samples are empty vector control amd ZF-552DNMT3a. (C) Proliferation analysis of MCF7 cells stable transduced with empty vector, ZF-552SKD, ZF-552DNMT3a, ZF-598SKD, ZF-598DNMT3a and DNMT3a. Stable cell lines were treated with Dox at time 0, after 48 hrs Dox was removed (indicated by *) and proliferation was followed for 192 hrs.

552DNMT3aE74A and ZF-598DNMT3aE74A) and untransduced control. The ZF-552DNMT3a methylated the SOX2 promoter at a higher level than the ZF-598DNMT3a. However, some background levels of methylation were detectable in the ZF-552DNMT3aE74A cells, which does not manifest in the ZF-598DNMT3aE74A cells.

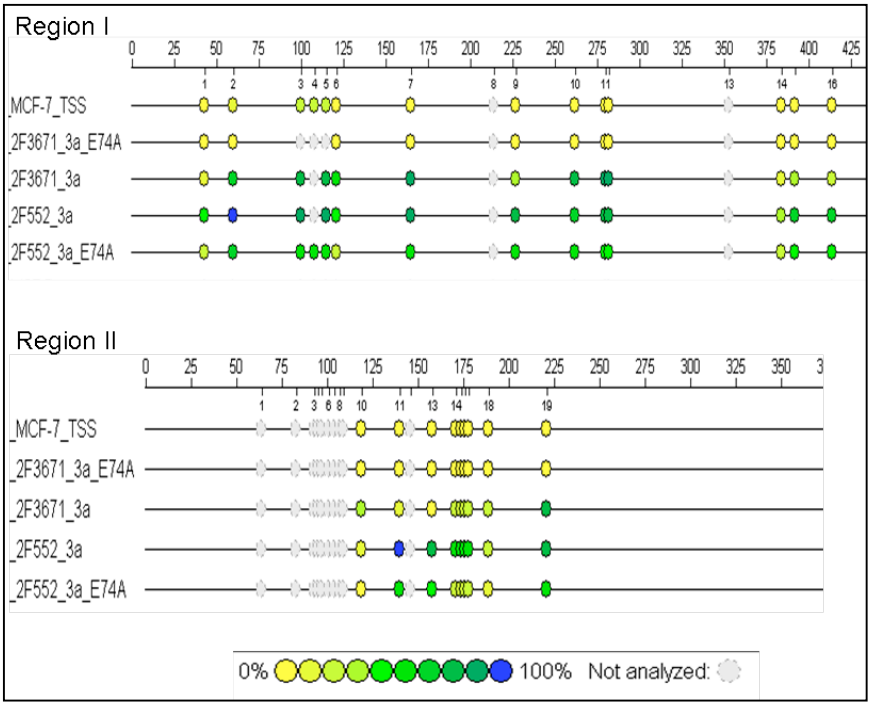

Fig. 7. DERFs promote targeted DNA-methylation analysed by MassARRAY analysis of 2 regions in the SOX2 promoter. MCF7 cells were retrovirally transduced with ZF-DNMT3a constructs or with their catalytic death mutant E74A. Two regions within the SOX2 promoter were analyses and percent in DNM-methylation is indicated in colour (0% = yellow to 100% = blue)

KEY RESEARCH ACCOMPLISHMENTS

Multimodular ZFPs designed to target the SOX2 oncogene in cancer cells down-regulate endogenous SOX2 expression.

Down-regulation of SOX2 expression inhibits tumor cell proliferation in vitro and in vivo.

DERFs (Designed Epigenetic Remodeling Factors) targeting SOX2, down-regulate SOX2 expression in mRNA and protein level.

Targeted incorporation of DNA methylation in the SOX2 promoter using the DNMT3a fused to two ZFP targeting different regions within the SOX2 promoter.

Targeted DNA-methylation decreases tumor cell proliferation in vitro more stable than transient transcriptional modifiers (SKD constructs).

ABSTRACTS AND MANUSCRIPTS

MANUSCRIPTS

Blancafort P, Juárez-Moreno K, Stolzenburg S, Beltran AS (2011) Engineering Transcription Factors in Breast Cancer Stem Cells. Breast Cancer - Carcinogenesis, Cell Growth and Signalling Pathways ISBN 978-953-307-714-7, InTech, Available from: http://wwwintechopencom/articles/show/title/engineering-transcription-factors-in-breast-cancer-stem-cells.

Rivenbark AG, Beltran AS, Yuan X, Rots MG, Strahl BD, Blancafort P. Epigenetic Reprogramming of Cancer Cells via Targeted DNA Methylation. *Epigenetics* (2012) Apr 1;7(4)

Stolzenburg S, Rots MG, Beltran AS, Rivenbark AS, Yuan X, Strahl BS, Blancafort P (2012) Targeted silencing of the oncogenic transcription factor SOX2 in breast cancer. *Nucleic Acids Research*. (2012) May 4 [Epub ahead of print].

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Blancafort P, Jin J, Frye S (2012). Writing and re-writing the epigenetic code of cancer cells: from engineered proteins to small molecules. *Mol Pharmacology* Minireview. *In Press*.

ABSTRACTS/PRESENTATIONS

Stolzenburg, S, Beltran, AS, Strahl, BS & Blancafort P. Epigenetic targeting of Sox2 using engineered ZF remodeling enzymes. Selected for a presentation in the Era of Hope Meeting, Orlando, Florida, August 2011.

Blancafort, P. Transcriptional regulation of breast cancer stem cell fate. American Society for Investigative Pathology. Breast Cancer Workshop. Washington, DC. April 13th, 2011.

Blancafort, P. Selection of Artificial Transcription Factors modulating breast cancer metastasis. Phage and Yeast Display Conference. Boston, MA. May 9th-10th 2011.

Blancafort P. Reprogramming Epigenetic Silencing with Artificial Transcription Factors. The Chilean Society for Cell Biology meeting. Pucon, Chile. October 29th –November 2nd 2010.

Blancafort P. Reprogramming Epigenetic Silencing with Artificial Transcription Factors. Department of Pharmacology, UNC. October 5th, 2010.

CONCLUSION

We confirmed previous findings that endogenous down-regulation of SOX2 expression in breast cancer cells lines decreases cell proliferation and tumor growth in xenograft mouse models. Furthermore, we fused ZF DNA-binding domains to the DNA-methyltransferase DNMT3a and engeneered catalytic impaired mutants (E74A). Delivery of ZF-DNMT3a constructs down-regulated endogenous SOX2 expression. This down-regulation was accompanied with a decreased cell proliferation in vitro. When compared to their transient counterparts (ZF-SKD), ZF-DNMT3a constructs exhibited a more stable

inhibition of cell proliferation. We demonstrated that DERFs incorporating direct DNA-methylation marks in endogenous promoters are powerful tools to reprogram breast cancer cells resulting in a decrease of cell proliferation with long-lasting effects.

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